RATE OF RESISTANCE DEVELOPMENT IN WILD *CULEX QUINQUEFASCIATUS* (SAY) SELECTED BY MALATHION AND PERMETHRIN

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Abstract. Wild caught female *Culex quinquefasciatus* (Say) from Kuala Lumpur were blood fed and reared in the insectarium. The late third stage of the F1 larvae which survived the high selection pressure of malathion and permethrin were reared and colonies were established from adults that emerged. Larvae from these colonies were then subjected in the subsequent 9 generations to higher selection pressure. The rate of resistance development were measured by LC_{50} value of larval bioassay, LT_{50} value of adult bioassay and the frequency of the elevated esterase levels. In another set of experiments using the same batch of *Culex* mosquitos, the larvae were not exposed to any insecticides and the decrease in resistance rate was monitored in each subsequent 9 generations by using similar methods. The heterozygous standard laboratory strain was selected for susceptibility using the single raft sib-selection method. The result showed that the field collected F1 generation was 96.0 and 6.3 fold more resistant to malathion and permethrin, respectively. After selection for about 9 generations the resistance ratio to malathion and permethrin was 6.2 and 767.3 fold more compared to the LC_{50} values of F1 generations, respectively. Esterase in F1 larvae was 6.0 fold more than the standard laboratory strain.

INTRODUCTION

Culex quinquefasciatus mosquitos are worldwide nuisance biting pests, and are vectors of urban filariasis and Japanese encephalitis. They breed and thrive abundantly in stagnant dirty water. In some countries their breeding sites have been sprayed with organophosphorous insecticides (Ketterman *et al,* 1993) and this has created the development of a resistance problem. Although there is no defined control program for *Culex* sp in Malaysia, this mosquito is highly resistant to organophosphates (Lee 1990; Lee *et al*, 1992). In Malaysia, the development of resistance could be due to the fogging operations with malathion in the early 1970s and with formulation containing permetrin in early 1996 against *Aedes* sp for dengue control. The development of resistance could have also enhanced by the large scale usage of insecticides in agricultural practices. Insecticide resistance is generally believed to arise from selection acting on random variation, *ie* pre-adaptive (Devonshire and Linda, 1991). However, it has been suggested that insecticides might act both by selection and by increasing mutation rates (Wood, 1984). The mechanism of resistance to organophosphorous insecticides involves the overproduction of non-specific esterases (Fournier *et al,* 1987; Mouches *et al,* 1987). The objective of this study was to determine the change rate of resistance development to the insecticides, malathion and permethrin, in the presence and absence of selection pressure and to determine the relationship of resistance and the non-specific enzyme esterase levels.

MATERIALS AND METHODS

Mosquitos

Adult *Culex quinquefasciatus* were collected from several localities in Kuala Lumpur and were bred in the insectarium of Division of Medical Entomology, IMR. The F1 and the subsequent larval stage generations were subjected to selection pressure. Standard susceptible laboratory(Penang) strain was used for comparison.

Insecticides

Malathion 93.3% ai (Cynamide) and permethrin 10.9% ai (Shell) were used in the study.

Selection pressure

The larval stages were subjected to selection pressure against malathion and permethrin at every

generation for at least 9 generations. The F1 and the successive generations of larvae and adult were used for the larval and adult susceptibility test. Larval and adult susceptibilities to these insecticides were determined by the WHO larval bioassay and adult bioassay test using dignostic dosages of malathion 5% and permethrin 0.25% (WHO, 1981 a,b) at each successive generation to obtain the 50% lethal concentration (LC_{50}) for larval stages and 50% lethal time (LT_{50}) for adults. Additional impregnated papers of 6% for malathion and 0.5% for permethrin were used against the resistant strain adults obtained from the larval selection pressure. A portion of the field caught F1 generation larvae was not exposed to any selection pressure and was bred in the usual manner in the insectarium. The larval and adult susceptibility was determined at each successive generation in order to study the rate of decrease in resistance in the absence of selection.

For selection of larvae, the insecticides were diluted in ethanol prior to adding into the 250ml water in the beakers used for the susceptibility test. The concentration chosen for the selection was one that gave 90-95% mortality. At least 1,000 larvae were exposed per insecticide per generation selection pressure for at least 9 successive generations. All selection pressure experiments were conducted under laboratory conditions of 28±1°C and RH of 85%. The changes in LC_{50} and LT_{50} were expressed as resistance ratios, calculated by dividing the final LC_{50} value of larval stage by the the starting LC_{50} at F1 for both malathion and permethrin. On the other hand, the starting LC_{50} value for the adult stage was at F3 generation for both the insecticides. The standard laboratory strain was found to be heterozygous for susceptibility. Hence, an attempt was made to obtain a homozygous susceptible strain using single raft sib-selection method (Tadano, personal communication). By using this method the susceptible mosquitos were isolated and reared for subsequent generations until pure susceptible strain were obtained. All bioassay results were subjected to probit analysis (Finney, 1971), using a computer program of Raymond (1985).

Rapid enzyme microassay

The rapid enzyme microassay was conducted according to the procedures of Lee and Tadano (1994). Individual female adult mosquitos and larvae were homogenized in 500 µl phosphate buffer and centrifuged at 14,000 rpm. The supernatant served as the enzyme source. Fifty microliters of the homogenate were transferred into each 8 wells in a microtiter plate. Fifty microliters of substrate solution were pipetted into each well and left for 60 seconds followed by the addition of 50 µl of coupling reagent. A deep purple color developed which turned to deep blue in room temperature on incubation for 10 minutes. The reaction was stopped by the addition of 50 µl of 10% acetic acid to each well. The intensity of the final color was indicative of the esterase levels. The intensity was read using an ELISA reader (Dynertac, 5000) at 450 nm.

The protein levels in the adults and larvae were also calculated according to Bradford (1976) method. The final esterase activity was expressed as nmoles of α-naphthyl acetate hydrolyzed /minute/ µg protein.

RESULTS AND DISCUSSION

The LC_{50} and LT_{50} of field collected *Cx. quinquefasciatus* larvae and adults, respectively, against malathion and permethrin is presented in Tables 1 and 2. The larvae and adult have been selected for 8 and 9 generations with malathion and permethrin, respectively. According to Darwinian theory, gene (s) responsible for insecticide resistance are existing in a small segment of the population. The gene (s) will be activated on exposure to insecticidal pressure. The speed and degree of development of resistance depends on the frequency of resistant genes in the population, the type of gene which is responsible for resistance, the insecticide dosage applied and the frequency of insecticidal application (Thomas, 1968).

From our results (Table 1) it was shown that the field strain, F 1 larvae were already resistant to malathion and permethrin, showing a resistance ratio of 96.2x and 9.4x, respectively in comparison with the sib-selection for the homozygous susceptible standard laboratory strain. However, after subjection to selection pressure with malathion (8 generations) and permethrin (9 generstions) the resistance ratio increased to 597x and 7,194x, respectively. It is obvious that permethrin resistance was developing at a higher rate compared to malathion (Fig 1). This trend supports a similar study in Cuba, where the *Cx. quinque-fasciatus* developed resistance to cypermethrin when this pyrethroid was used in alternate cycles with

Table 1

LC_{50} value of malathion and permethrin selection pressure aganinst *Culex quinquefasciatus* larvae.

	LC_{50} (mg/l)			
	Malathion		Permethrin	
Generation	Nemal	Malathion	NEperm	Permethrin
F1	1.25	1.25	0.015	0.015
	(0.60)	(0.60)		
F2	1.40	-	0.15	-
	(0.26)			
F3	2.49	0.42	0.08	0.004
	(0.45)	(0.47)*	(0.45)	(0.50)*
F4	0.22	0.84	0.35	0.73
	(0.77)	(0.77)*	(0.77)	(0.80)*
F5	0.23	15.27	0.02	0.73
	(0.74)	(0.75)*	(0.77)	(0.80)*
F6	4.44	3.13	0.03	2.40
	(0.51)	(0.48)*	(0.51)	(0.42)*
F7	0.35	1.35	0.04	7.11
	(0.19)	(0.50)*	(0.29)	(0.19)*
F8	0.24	7.76	0.01	9.68
	(0.21)	-	(0.21)	(0.40)*
F9	0.41	-	0.03	11.51
	(0.22)	-	(0.22)	(0.35)*
Susceptible strain	-	0.013	-	0.0016
		(0.10)		(0.10)

NEmal and NEperm : non exposed larvae tested with malathion and permethrin respectively.
(parentheses) : indicates esterase level (nmoles of α - naphthyl acetate hydrolyzed /minutes /μg protein).
*significance of NE esterase activity in larvae to malathion and permethrin selection using Student's *t*-test at $p < 0.05$.

malathion (Rodriguez *et al*, 1993). Bisset *et al*, (1997), reported high resistance development in *Culex* against the pyrethroid, lamda cyhalothrin, after 6 generations of selection and cross-resistance was observed against malathion. Similarly, Kang *et al*, (1995), observed unequivocal resistance to deltamethrin when subjected to selection.

Comparing the F1 LC_{50} value of malathion and permethrin to their LC_{50}'s of their respective generations of selection, the resistance ratio was 6.2x and 767x, respectively. Studies by Bisset *et al* (1991) and Gopalan *et al* (1996) demonstrated 1,208 fold resistance after 22 generations and 2,036 fold resistance after 25 generations of selection with malathion. It was not possible to calculate the rate of selection in each generation due to the inconsistency in the larval LC_{50} values which could be due to heterozygosity and homozygosity of the gene(s). It should be noted that if selection pressure is removed from the field population for 9 generations *eg* in our study, there is no drastic decrease in resistance against malathion and permethrin, *ie* 3.1x and 5.0x, respectively. In contrast, Rodriguez *et al* (1993), reported malathion resistance to *Cx. quinquefasciatus* declined when the pyrethroid, cypermethrin was used alternatively with malathion in the period of 4 years, 1987-1991.

Adult susceptibility levels were assessed with 5% and 0.25% WHO diagnostic dosage impregnated papers of malathion and permethrin, at 1 hour and 3 hours exposure, respectively. Mortality was also recorded after 24 hours. Additional dosage of 6% and 0.5% impregnated papers for malathion and

Table 2

LT_{50} value of malathion and permethrin selection pressure aganinst *Culex quinquefasciatus* adult.

	LT_{50} (min)			
	Malathion		Permethrin	
Generation	NEmal	Malathion	NEperm	Permethrin
F1	-	-	-	-
F2	-	-	-	-
F3	58.79	> 60**	42.92	302.4
	(0.67)	(1.02)*		
F4	182.64	90.30**	105.79	> 180
	(1.38)	(1.14)*	(1.38)	(0.97)*
F5	64.24	75.39**	154.74	>180
	(1.41)	(1.38)*	(1.41)	(1.46)*
F6	67.39	> 60**	100.58	> 180
	(0.56)	(0.94)*	(0.56)	(0.73)*
F7	50.57	> 60**	111.35	> 180
	(0.56)	(0.79)*	(0.56)	(0.73)*
F8	71.34	> 60	69.59	> 180
	(0.59)	(0.82)	(0.59)	(0.67)*
F9	77.46	> 60	71.84	262.93**
	(0.67)		(0.67)	(0.79)*
Susceptible strain	-	16.46	-	24.42
		(0.29)		(0.29)

NEmal and NEperm : non exposed larvae tested with malathion and permethrin respectively.
(parentheses) : indicates esterase level (nmoles of α - naphthyl acetate hydrolyzed /minutes /μg protein).
*significance of NE esterase activity in adult to malathion and permethrin selection using Student's *t*-test at $p < 0.05$.
** was tested with 6% and 0.5% malathion and permethrin impregnated papers, respectively.

permethrin were used for the succesive selection pressure resistant generations of malathion and F 9 generation of permethrin, respectively. Our results indicated that 100% survival was obtained for both the malathion and permethrin selection pressure at F 3 to F 9 generations, respectively. At 24 hours, 44.5 - 100% and 0-86.6% mortality were observed in the non- selected malathion, and malathion selected strains. The non-selected permethrin and permethrin selected strains showed 48.9-100% and 0-33.3% mortality, respectively (Fig 2). In the absence of selection against malathion and permethrin, both strains showed a 2-fold decrease in resistance in the 24 hours mortality data (Fig 2). From our study it is also obvious that resistance gene(s) expression was more active in larvae compared to adults in comparison with the resistance ratio results of malathion and permethrin and the mortality at 24 hours. Similarly, Tadano and Brown (1966) reported a lower resistance ratio in adults compared to larvae after subjection to several insecticides. adult from non- selected strains showed LT_{50} values in the range 58.8 - 77.46 minutes and the decrease in each succesive generations was not consistent for malathion. In contrast, against permethrin, there was an approximately 5 times (F9/ F2) reduction in LT_{50} values.

In addition to the bioassays, the esterase levels were also used to measure the degree of resistance. The larval esterase levels demonstrated activity of 6 fold greater in comparison with the susceptible strain. The larvae of malathion and permethrin selection strains exhibited rather fluctuating esterase levels, whereas in the non-exposed mala-

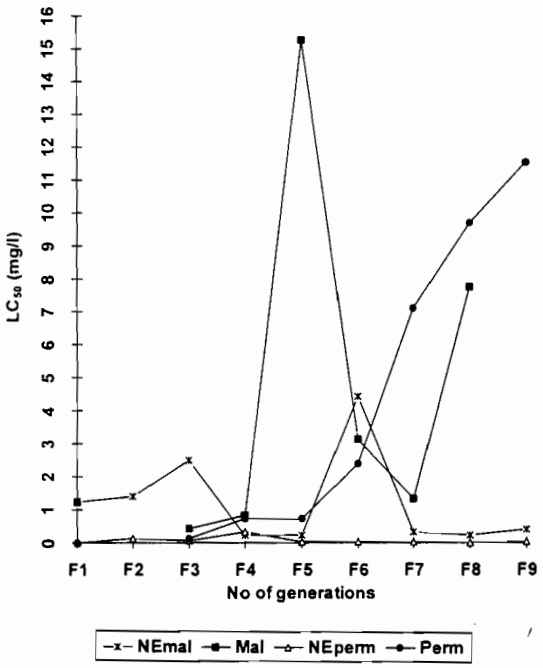

Fig 1–LC_{50} value of malathion and permethrin for NEmal, mal, NEperm and permethrin at larval stages for all geneations.

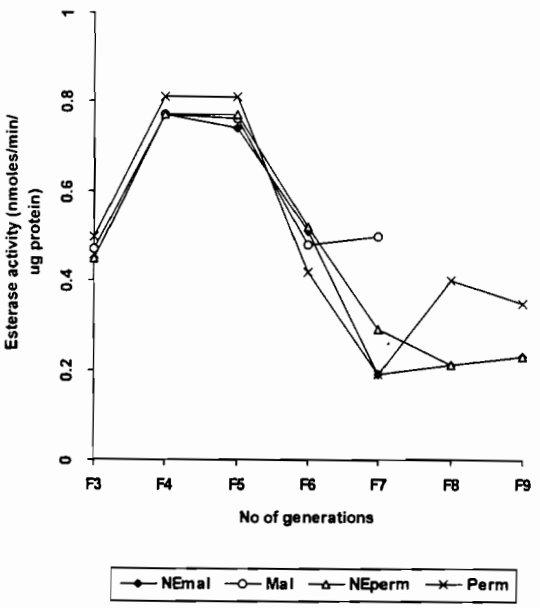

Fig 3–Larval esterase activity in the non- selected (NE) and selection pressure strains against malathion and permethrin.

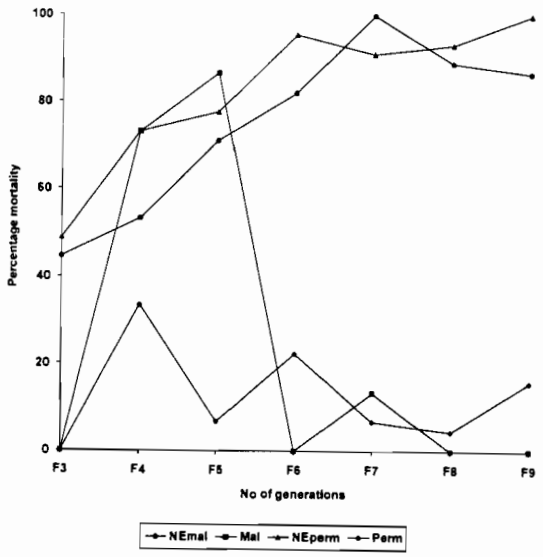

Fig 2–Twenty four hours bioassay mortality for every generation for adults of NEmalathion, malathion, NEpermethrin and pemethrin seiection pressure strains.

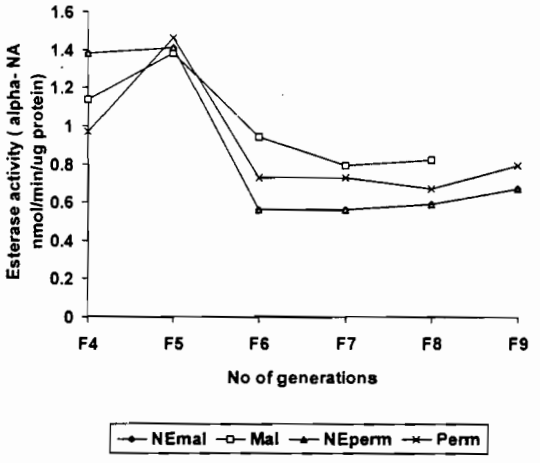

Fig 4–Adult esterase activity in the non-selected (NE) and selection pressure strains against malathion and permethrin.

thion and permethrin strains, the results showed 3 and 2 fold reduction in enzyme activity respectively (Fig 3). Similar trends were observed in the selected and non-selected strains of adults (Fig 4).

From the above study, it clearly showed permethrin selection for resistance was at a faster rate compared to malathion based on the LC_{50} values. The esterase level was not significantly high in the selection pressure strains. Hence, there was no clear relationship between the bioassay and rapid enzyme microassay results.

The information obtained in this study is useful in mosquito control programs and may help in the development of strategies to overcome insecticide resistance. The present control program for *Aedes* mosquitos is the use of permethrin, perhaps this has to be reconsidered because, permethrin induces resistance at a faster rate compared to malathion.

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